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Tumor Necrosis Factor Alpha Levels in Plasma and Whole-Blood Culture in Dengue-Infected Patients: Relationship Between Virus Detection and Pre-existing Specific Antibodies

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The pathogenesis of dengue hemorrhagic fever (DHF) is not well known, but the role of host factors has been suggested. The level of immunoreactive circulating and cell-generated tumor necrosis factor alpha (TNFα) was studied in 35 patients with DHF; its relationship with virus isolation and/or genome detection by reverse transcription polymerase chain reaction (RT-PCR) and specific antibodies were detected by hemagglutination inhibition (HI). Large variation of TNFα plasma levels was obtained in dengue-infected patients at the same stage of the disease and at the same day after infection. Most of the patients (14 out of 17 patients) who displayed augmented spontaneous in vitro production of TNFα by heparinized whole-blood culture compared with controls also had elevated levels of TNFα in the plasma. The TNFα values in lipopolysaccharide and phytohemagglutinin heparinized whole-blood cultures were not higher in patients than in controls, but low TNFα levels were obtained in three out of 30 patients. An inverse correlation was observed between spontaneous in vitro TNFα production and viral replication, which raises the issue of the antiviral effect of TNFα in dengue infection. The results do not support the hypothesis of the role of antibody-dependent enhancement giving rise to increased viremic titers and production of TNFα in patients. The present study demonstrates the activation of the TNFα-producing cells in dengue-infected patients and suggests further investigation to define the mechanism and the role of TNFα in the pathogenesis of dengue virus infection. ***J. Med. Virol. 54:210–218, 1998.***

KEY WORDS: Vietnam; virus isolation; RT-PCR; anti-dengue virus antibodies

INTRODUCTION

Dengue fever is a common childhood disease in tropical countries that has been endemic for many years primarily in some parts of south east Asia and has spread to intertropical areas [Monath, 1994]. Although dengue virus infection (four serotypes in the flavivirus genus of the Flaviviridae family) usually results in mild disease, sometimes infection by this virus results in a severe disease such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [Cohen and Halstead, 1966]. The pathogenesis of dengue is incompletely understood, but some evidence suggests that an abnormal immune response and a disturbance in immune regulation may be responsible for the pathogenesis of shock and excessive hemorrhage in patients with DHF/DSS [Halstead, 1989; Pang, 1987; Suvatte, 1987]. Cytokines are involved in immune regulation but could play a role in pathologic manifestations of dengue infection [Halstead, 1989]. Tumor necrosis factor alpha (TNFα) is a pluripotent polypeptide cytokine mainly produced by monocytes, macrophages, and T-lymphocyte subsets which is involved in numerous

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pathologic phenomenon such as septic shock and inflammation and which could play a role in the pathophysiology of dengue virus infection [Tracey et al., 1988; von Asmuth et al., 1990].

It has been suggested that monocytes/macrophages are activated in dengue-infected patients [Halstead, 1989]. However, reports concerning circulating levels of the monocyte-derived cytokine TNFα in dengue-infected patients are conflicting [Hober et al., 1993; Kuno and Bailey, 1994; Kurane et al., 1991; Vitarana et al., 1991; Yadav et al., 1991]. If cells from dengue-infected patients are activated in vivo, they should show some markers or functions associated with the activation state. These observations together with the studies indicating the importance of TNFα in dengue virus infection have led us to investigate the production of TNFα in patients at different stages of infection. To our knowledge, simultaneous measurement of circulating and cell-generated TNFα in dengue-infected individuals has not been reported.

The different states of the cells may be evaluated by their ability to produce cytokines in vitro. Although the production of cytokines is usually studied on isolated peripheral blood mononuclear cells (PBMCs), whole blood is being used frequently [Allen et al., 1992; Wilson et al., 1991]. The measurement of cytokines in whole-blood culture is a simple and rapid procedure that reduces the risk of contamination by endotoxin during isolation. Because it reflects the natural environment, we estimate that whole-blood culture is the appropriate medium for studying TNFα production in vitro.

Epidemiological evidence suggested that prior immunity to dengue virus could play a role in the pathogenesis of DHF, but this is a subject of controversy since not all individuals with preinfection antibodies experience DHF or DSS during subsequent dengue infection [Rosen, 1989]. Pre-existing antibodies in serum can enhance infection of monocytes with dengue virus by an antibody-dependent mechanism [Morens and Halstead, 1990]. Recently it has been reported that antibody-enhanced in vitro infection of dengue virus in monocytes induced TNFα which activates endothelial cells. A much higher activation was obtained with dengue virus and antibodies rather than with virus alone [Anderson et al., 1997]. TNFα can initiate events involved in endothelial dysfunction, like vascular leakage, which may play a role in DHF/DSS. These data suggest that virus-specific antibodies may increase production of TNFα in dengue-infected subjects which may facilitate the development of DHF/DSS.

It has been reported that a direct productive infection of monocytes with dengue virus resulted in an increase in the secretion of TNFα by these cells [Chang and Shaio, 1994] and that PBMCs from donors challenged with dengue virus in vitro released large amount of TNFα [Yang et al., 1995].

The significance of these observations relative to in vivo pathogenesis remains unclear. Therefore, the relationship between TNFα level, virus detection, and pre-existing dengue virus-specific antibodies was studied in dengue-infected patients.

PATIENTS, MATERIALS, AND METHODS

Patients

The study was carried out in 35 children admitted to the Dengue Hemorrhagic Fever Department of Pediatric Hospital No. 1, Ho Chi Minh City, Vietnam. The World Health Organization (WHO) categorized DHF cases into four grades from less severe (grade I) to severe (grade IV) [World Health Organization, 1980]: Grade II is characterized by fever associated with nonspecific constitutional symptoms and spontaneous hemorrhagic manifestations; grade III is demonstrated by circulatory failure manifested by rapid, weak pulse with narrowing of the pulse pressure (<20 mm Hg) or hypotension; and grade IV is characterized by deep shock with undetectable blood pressure and pulse. The patients ranged from 3 to 15 years of age; there were 16 patients classified as grade II, 18 as grade III, and one as grade IV. Children without any infectious disease, who either were hospitalized or were outpatients at Pediatric Hospital Ho Chi Minh City (n = 22), were included as controls. Their parents gave their consent for drawing blood. The patients were admitted to the hospital on the indicated date postinfection (see Table 1 in the results section). Most of grade III patients and the grade IV patient were admitted to the hospital the day after the shock occurred. The diagnosis was determined clinically and was confirmed by laboratory tests.

Blood Samples

Blood was drawn into sterile vacuum collection tubes and into sterile tubes containing EDTA (Vacutainer, Becton Dickinson, Meylan, France) and sterile Eppendorf tubes containing endotoxin-free sodium-heparin (PanPharma, France). Blood samples were collected for each patient on admission to the hospital and during hospitalization. For a given subject, one single sample of heparinized whole blood was obtained on admission to the hospital; at the same time, a first sample of EDTA-treated whole blood was collected, and then additional EDTA-treated blood samples were collected consecutively for 2 days. Plasma was separated within 1 hour after collecting of EDTA-whole blood, frozen at −70°C, transported to the laboratory of viral diseases at University Hospital of Lille, France in dry ice, and stored at −70°C until use. Within 1 hour after collection the blood was processed as described below. Serum was separated 1 hour after collecting of blood and processed for dengue virus isolation and serological tests.

Virus Isolation and Virus Genome Detection

Dengue virus isolation by inoculation of C6/36 cells. Virus isolation were carried out at the Pasteur Institute of Ho Chi Minh City. Subconfluent *Aedes albopictus* C6/36 cell monolayers were inoculated with 1/10 dilution of serum samples for 1 hr, washed with Dulbecco's minimum essential medium (DMEM), and incubated for 7 days at 28°C in DMEM supplemented

TABLE I. TNFα Plasma Levels (pg/ml) in Dengue Virus–Infected Patients

Grade (WHO)	Patient	Days after the onset of the disease[a] D3	D4	D5	D6	D7
II						
	a	45	51	54		
	b		47	36	29	
	c			155	41	55
	d			61	41	0
	e			33	26	30
	f		311		33	39
	g			348	8	6
	h			141	244	241
	i			9	28	32
	j	21	89	45		
	k	313	418	520		
	l			373	164	303
	m			66	35	20
	n		77	73	37	
	o			52	30	30
	p		1000	705	332	
III						
	a′		27	37	60	
	b′			1,000	170	74
	c′		0	100	51	
	d′			72	36	200
	e′			28	21	25
	f′			621	69	266
	g′			200	255	252
	h′		31	23	28	
	i′			43	37	42
	j′			132	494	421
	k′			138	121	117
	l′			85	39	50
	m′			36	81	nd
	n′			36	37	38
	o′			57	89	38
	p′			54	44	37
	q′			35	31	37
	r′		39	29	39	
IV						
	s′			40	28	43

[a]Blood was drawn into sterile tubes containing EDTA. Sequential blood samples were obtained consecutively for 3 days from the admission at the Pediatric Hospital No. 1, Ho Chi Minh City, Vietnam. The mean + 2 SD of 12 controls was 38 pg/ml. nd, not done.

with 10% fetal calf serum. Cells were washed with PBS, gently resuspended, and deposited on an immunofluorescent slide. Serotype-specific dengue antigens in infected cells were detected by an indirect immunofluorescence assay (IFA) using type-specific mouse monoclonal antibodies and fluorescein-labeled anti-IgG [Henchal et al., 1983].

Virus detection and identification by RT-PCR. In the EDTA-plasma of certain patients dengue virus was detected by using RT-PCR, performed at Pasteur Institute of Paris. RNA from 10 μl of plasma samples was extracted using the procedure described previously [Deubel et al., 1997]. The RT-PCR method was a modification from that described previously by Lanciotti et al. [1992]. Twenty nanograms of the complementary D2 primer (5′-TTGCACCAACAGTCAATGTCTTCAGGTTC-3′) was used to prime the synthesis of cDNA of target RNA with six units of avian myeloblastosis virus reverse transcriptase (Promega) in 20 μl of buffer (10 mM Tric-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ containing 0.2 mM of each of the four dNTPs, and 10 units of rRNasine). The mixture was incubated for 1 hr at 42°C. The amplification reaction was performed by combining the cDNA product and an amplification mix containing the following components: PCR buffer (Promega, 2.5 mM $MgCl_2$, 0.5 mM dNTPs, 300 ng of each of primers D2 and genomic sense D1) (5′-TCAATATGCTGAAACGCGC-GAGAAACCG-3′), and 2 units of Taq polymerase (Promega). Target cDNA was amplified in 50 μl volume in a thermocycler programmed to denature 3 min at 94°C and then to proceed with 20 cycles of denaturation (94°C, 30 sec), primer annealing (53°C, 90 sec), and primer extension (72°C, 1 min). A second amplification reaction was performed with 10 μl from 1:100 dilution of the initial amplification product by replacing primer D2 with the dengue virus type-specific primers TS1 (5′-CGTCTCAGTGATCCGGGGG-3′), TS2 (5′-CGCCACAAGGGCCATGAACAG-3′), TS3 (5′-TAACATCATCATGAGACAGAGC-3′), and TS4 (5′-CTCTGTTGTCTTAAACAAGAGA-3′) described previously [Lanciotti et al., 1992]. The PCR products were analyzed by electrophoresis on a 4% agarose gel, and amplified DNA fragments were visualized by staining the gel with ethidium bromide. The size of the resulting DNA band was characteristic for each dengue virus type, i.e., 482 bp for dengue 1, 119 bp for dengue 2, 290 bp for dengue 3, and 392 bp for dengue 4.

Serological Test

The serodiagnosis was based upon seroconversion between early and late serum samples by hemagglutination inhibition carried out at the Pasteur Institute of Ho Chi Minh City. Briefly, serum samples were extracted with acetone, adsorbed with goose erythrocytes, and tested for hemagglutination-inhibiting antibodies against the four dengue antigens as previously described [Clarke and Casals, 1958]. Titers correspond to the inverse of serum dilution. Titers = 160 against all the four dengue antigens at the first serum sample are considered as secondary infection.

Whole-Blood Culture

Heparinized whole blood (25 μl) was transfered to sterile 96-well plates (Nunc) containing 45 μl endotoxin-free and mycoplasma-free RPMI 1640 (Gibco-BRL) in duplicate in the absence or in the presence of lipopolysaccharide (LPS), 25 μg/ml (LPS from Salmonella Enteriditis, Sigma, St. Louis, MO) and phytohemagglutinin (PHA), 5 μg/ml (PHA HAH from Wellcome Diagnostics, Dartford, UK). The plates were incubated for 24 hr at 37°C in a 5% CO_2 air atmosphere. Then the supernatants of the blood cultures were harvested and pooled. They were frozen at −70°C, transported to the laboratory of viral diseases at University Hospital of Lille, France in dry ice, and stored at −70°C until use.

TNFα Measurements

The concentrations of TNFα in the plasma and supernatants of cultures were assayed with the TNF = EASIA

kit (Medgenix Diagnostics SA, Fleurus, Belgium). This assay is based on the oligoclonal system in which several monoclonal antibodies (MAbs) directed against distinct epitopes of the cytokine are used. Soluble TNF receptors (sTNFRs) do not interfere with TNFα-EASIA assay. The assay was performed according to the instructions of the manufacturer. The microtiter plates were read at the appropriate wavelength. The absorbance was proportional to TNFα concentration, which was determined by reading a standard curve. Intra-assay variation was less than 5%, and interassay variation was less than 10%. The minimum detectable concentration of TNFα was 3 pg/ml. Each value was defined as the mean of two determinations.

Analysis of Data

The statistical significance of the differences of TNFα levels in patients and controls was evaluated by the Student's t-test. χ^2 analysis of the results was conducted when appropriate.

RESULTS

Follow-Up of Plasma Level of TNFα in Dengue-Infected Children

The variations of TNFα level in sequential blood samples obtained consecutively for 3 days from admission to the hospital are shown in Table 1. The mean ± SEM of 12 control children was 30.75 ± 1.1 pg/ml, and the mean + 2 SD was 38 pg/ml. We obtained a large variation of TNFα in dengue-infected patients at the same grade of the disease and at the same day after infection. Stable levels lower than the mean + 2 SD of controls were observed in some patients of grade II and grade III (patients e, e′, h′, n′, q′). High levels of TNFα with decreasing values from the first to the third sample were obtained in six patients (c, d, f, g, p in grade II and b′ in grade III). Stable high levels in three consecutive samples were obtained in six patients (patients h, k, l in grade II and patients g′, j′, k′ in grade III). The highest values (1,000 pg/ml) were observed in the first plasma sample from a grade II patient (patient p) and in the first plasma sample from a grade III patient (patient b′) on day 4 and day 5 post-onset of the disease, respectively.

Production of TNFα by Whole Blood From Dengue-Infected Children

The presence of high levels of TNFα in circulating blood from some dengue-infected patients is in favor of the activation of the TNFα-secreting cells in the course of the disease. To answer this question whole blood was collected from dengue-infected patients when they were admitted to the hospital. Heparinized blood was incubated for 24 hr, then the supernatant of culture was harvested to evaluate the spontaneous production of TNFα. The results presented in Figure 1B and B′ demonstrate that the blood from certain dengue-infected individuals spontaneously release high levels of TNFα, in contrast to HWB from controls that produce little TNFα. Higher values of TNFα than the mean + 2 SD of controls (142 pg/ml) were obtained in seven out of 14 grade II patients and in ten out of 16 grade III/IV patients. When the spontaneous production of TNFα and the circulating level of TNFα were compared at the same time in 30 patients, we found high values of both parameters in 14 patients and normal values of both parameters in five patients; however, a discordance was observed between those parameters in certain patients. Indeed in eight patients high values of TNFα were obtained in plasma associated with normal production of TNFα in culture, and in three other patients normal TNFα plasma levels were associated with high production of the cytokine in culture.

The activation state of the cells in peripheral blood of the patients with dengue infection was investigated further when they were admitted to the hospital. Blood was cultured for 24 hr in the presence of LPS and PHA, and then the supernatants were harvested for TNFα measurement. In ten controls the TNFα values were 19,257 ± 7,517 pg/ml, ranging from 7,870 to 27,920 pg/ml. In most of the patients we obtained an increased production of TNFα in cultures with stimulant compared with cultures without stimulants; the values ranged between the mean − 2 SD and the mean + 2 SD of controls, and the mean ± SD values of grade II and grade III patients were 19,957 ± 11,552 n = 14 and 13,847 ± 7,531 n = 16, respectively (*P* values not significant vs. controls) (Fig. 2). In three patients, the concentrations of TNFα in activated cultures were lower than the mean − 2 SD of controls (4,223 pg/ml); the individual values were 1,619 pg/ml (grade II), 2,849, and 2,488 pg/ml (grade III), respectively (mean ± SD value: 2,318 ± 632 pg/ml n = 3, *P* value .003 vs. controls). In those grade III patients the spontaneous production of TNFα was normal. The differences in TNFα values between those three patients and the remaining patients in this study cannot be due to the number of cultured cells since no correlation was found between the white cell counts in peripheral blood and the TNFα values in supernatant of culture (data not shown).

Spontaneous In Vitro Production of TNFα and Detection of Dengue Virus in Peripheral Blood

To investigate a possible relationship between TNFα levels and the outcome of the disease, TNFα values in patients were compared with the isolation of dengue virus from the serum by culture and/or the detection of its genome by RT-PCR. Blood for virus isolation and genome detection was collected in patients when they were admitted to the hospital. Dengue 2 virus was found by viral culture and/or RT-PCR in 12 patients (group 1), and the assays were negative in 15 other patients (group 2). It was found that the spontaneous in vitro production of TNFα was significantly lower in most patients in whom virus was detected in their peripheral blood (61 ± 71 pg, n = 10) than in most patients in whom virus was not detected in their peripheral blood (3,064 ± 3,455 pg/ml n = 11) (see Table 2) (*P*

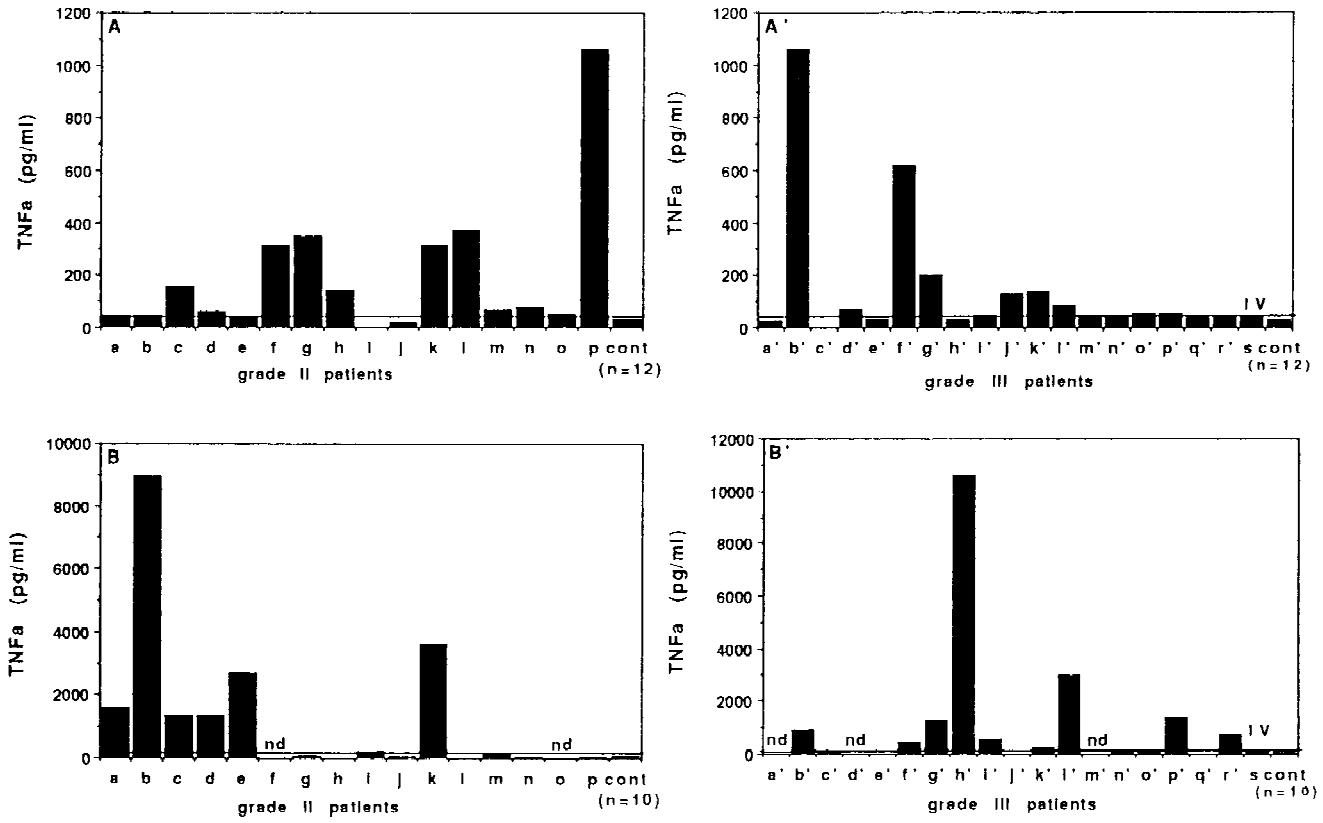

Fig. 1. Individual representation of plasma levels of tumor necrosis factor alpha (TNFα), and spontaneous production of TNFα in heparinized whole-blood culture in patients with dengue-virus infection grouped according to WHO criteria as grade II (n = 16), grade III (n = 18), and grade IV (n = 1). Each bar represents one subject. Every parameter was determined with blood samples collected at the same time (the admission day at the hospital). Heparinized whole blood was cultured for 24 hr as described in Patients, Materials, and Methods. **A, A′:** TNFα plasma concentrations in pg/ml in grade II and grade III and IV patients, respectively. **B, B′:** TNFα concentrations in pg/ml in supernatant of heparinized whole-blood culture of grade II patients and grade III and IV patients, respectively. Label IV in A′ and B′ indicate the grade IV patient (patient s′) included in this study. The mean ± SEM of plasma values of TNFα in 12 controls designated as "cont" was 30.75 ± 1.1 pg/ml. The mean ± SEM of TNFα concentrations in pg/ml in supernatant of heparinized whole-blood culture in ten controls designated as "cont" was 65.7 ± 12 pg/ml. The lines represent the mean + 2 standard deviations (SD) of plasma values of TNFα in 12 controls (38 pg/ml) (A, A′) and the mean + 2 SD of TNFα concentrations in supernatant of heparinized whole-blood culture in ten controls (142 pg/ml) (B, B′), respectively. nd, not done.

= .0015, χ^2 test). However, a clear correlation was not found between spontaneous production of TNFα in vitro and the plasma levels of TNFα in groups 1 and 2. Indeed, no significant difference was found between plasma levels in low and high TNFα producers. The mean plasma levels in patients with low TNFα production in group 1 (217 pg/ml, n = 10) and in patients with high TNFα production in group 2 (223 pg/ml, n = 11) were higher than the mean plasma level of controls (30 pg/ml, n = 12), but the difference vs. controls was not statistically significant due to the range of values. No correlation was found between TNFα level and virus detection in any of the disease grades. Indeed, viremic patients showed a normal production of TNFα in vitro in four grade II and six grade III patients. Moreover, in patients in whom virus was not detected a high production of TNFα in vitro was found in six grade II and five grade III patients (see Table II).

Spontaneous Production of TNFα and Anti-Dengue Antibodies

The TNFα values were compared with the isolation of dengue 2 virus from the serum by culture and/or the detection of its genome by RT-PCR and with the presence of anti-dengue 2 virus antibodies in patients when they were admitted to the hospital (see Fig. 3). Primary infection was defined as seroconversion between early and late samples and HI titers < 160 (i.e., 0, 20, 40, 80) for the four dengue antigens in the first serum sample. High TNFα levels in vitro were obtained in five out of 11 patients with primary dengue infection and in eight out of 16 patients with secondary dengue infection. Normal TNFα levels in vitro were obtained in six out of 11 patients with primary dengue infection and in eight out of 16 patients with secondary dengue infection. High TNFα levels were obtained in two patients without antibodies for dengue 1, 2, 3, and 4 antigens detected by HI. Thus, there was no apparent correlation between the in vitro production of TNFα and the presence of specific antibodies in dengue-infected patients. In addition, the plasma levels of TNFα were not correlated with the presence of specific antibodies (data not shown). In patients with detectable viremia, eight out of 12 patients were found to have secondary infection, and in patients without detectable viremia eight out of

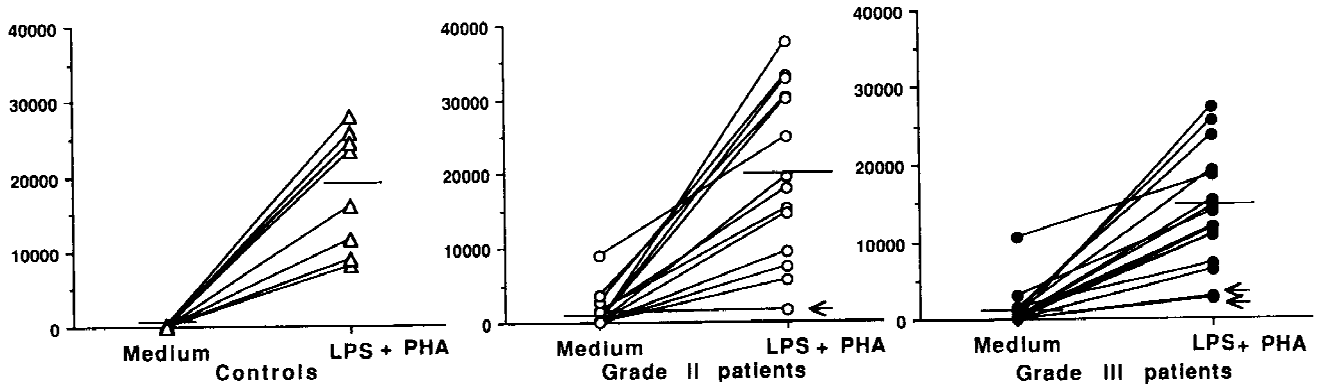

Fig. 2. Individual representation of stimulated TNFα release in dengue-infected patients blood. Heparinized whole blood was cultured for 24 hr in the presence of medium or LPS (25 μg/ml) and PHA (5 μg/ml) as described in the Materials and Methods. Then the supernatants were harvested for TNFα measurements by enzyme-induced immunosorbent assay ELISA. The horizontal bars represent the mean of TNFα values. The arrows indicate the patients with concentrations of TNFα in stimulated cultures lower than the mean −2 SD of controls. Controls: n = 10; grade II patients, n = 14; grade III patients, n = 16.

15 patients were found to have secondary infection. Therefore, no correlation between viremia and the presence of specific antibodies in dengue-infected patients could be established.

DISCUSSION

Increased levels of TNFα were found in dengue 2 infection in southeast Asia in agreement with a recent report of an increase of TNFα in dengue 3 infection in Tahiti [Hober et al., 1993].

In other studies, an increase of TNFα in dengue-infected patients was described [Kuno and Bailey, 1994; Vitarana et al., 991; Yadav et al., 1991]; however, our study is different in many respects from those of other investigators: First, the plasma level of TNFα was studied in each patient; second, the plasma levels of endogenous TNFα were investigated in the same patients whose blood was used for in vitro production of the molecule; third, the relationship between TNFα levels, viral parameters, and immunological parameters were studied.

The plasma samples obtained from certain patients at one time point but not those obtained at other time points showed high levels of TNFα. In other patients, the TNFα levels were normal. The discrepancy in the results obtained in different studies [Kurane et al., 1991] can be explained in part by the variation in the level of TNFα during the course of dengue infection, by the rapid clearance of TNFα from the circulation, by immunological differences, and, as in studies of HIV infection, may to a considerable extent be related to the techniques used for measuring TNFα [Aukrust et al., 1994]. The possibility that sTNFR may have interfered with the determination of TNFα in our samples can be excluded, as the TNFα immunoassay that we used can detect TNFα in complex with its soluble receptors and TNFα monomers and polymers as well [Aukrust et al., 1994].

In most grade III patients the blood samples were obtained 1 day after the shock occurred; therefore, the TNFα concentrations could be higher on the 1st day postinfection than those detected in the plasma obtained at the hospital. Indeed, in a previous report we showed that TNFα concentrations decreased 1st day after the diagnosis of shock [Hober et al., 1993]. However, it was reported that circulating TNFα may signal only the presence of inflammatory stimulus but is not a quantitative measure of the host reaction. Indeed, the localized cytokine level is more indicative of the inflammatory status than the cytokine level in the plasma or serum [Sekut et al., 1994].

The in vitro production of cytokines is usually studied on isolated peripheral blood mononuclear cells. However, the isolation procedure eliminates polymorphonuclear cells that may play a role in intercellular communications regulating cytokine release, may lead to cell activation and to a change in the ratio between the cells, and may deprive cells of important factors present in whole blood [Degroote et al., 1992]. Therefore, we used whole blood culture for studying TNFα production in vitro, using a method which requires a small volume of blood.

The extent of the increase of TNFα levels in culture compared with those in plasma demonstrates that the TNFα detected is not residual but is released by cells in the course of the culture. The role of contaminant endotoxin in the high production of TNFα in blood culture from patients could be ruled out since the culture of blood from controls under the same conditions did not lead to an increased production of TNFα.

The results confirm the activation of the TNFα-producing cells in dengue-infected patients. High levels of TNFα were found in plasma and/or supernatant of culture of heparinized blood in 25 out of 35 patients. Most of the patients who had augmented spontaneous in vitro production of TNFα by heparinized blood also had elevated levels of endogenous TNFα in the plasma.

A rapid degradation of TNFα in circulating blood may occur; in addition, this cytokine may be released and consumed locally at the site where the immune

TABLE II. TNFα Level and Virus Detection in Dengue-Infected Patients

		High				Normal			
				Grade (WHO)				Grade (WHO)	
	N	TNFα level in vitro (pg/ml)[a]	Plasma level of TNFα (pg/ml)	II n	III n	TNFα level in vitro (pg/ml)	Plasma level of TNFα (pg/ml)	II n	III n
Group 1 Virus isolation and/or RT-PCR$^+$	12	2,116 ± 1221 ($P < .001$)[b]	142 ± 81 ($P < .001$)	2	0	61 ± 71 ($P = .08$)	217 ± 325 ($P < .05$)	4	6
Group 2 Virus isolation and/or RT-PCR$^-$	15	3,064 ± 3455 ($P < .02$)	223 ± 331 ($P > .05$)	6	5	58 ± 47 ($P = .7$)	79 ± 44 ($P = .001$)	3	1

[a]Spontaneous production of TNFα in heparinized whole-blood culture is obtained as described in Materials and Methods. Patients are grouped according to WHO criteria. High, high spontaneous TNFα level; normal, normal spontaneous TNFα level.
[b]*P* values versus dengue-negative controls.

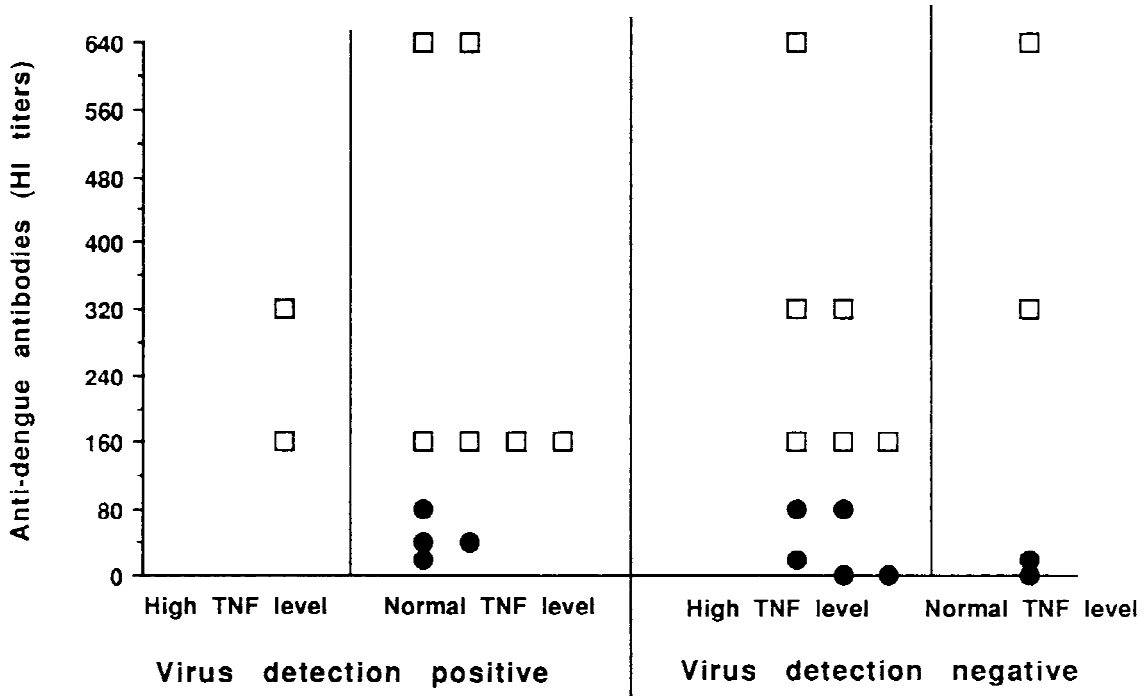

Fig. 3. Individual representation of anti-dengue antibody titers in dengue-infected patients grouped according to the presence of dengue virus or its genome in their peripheral blood and the spontaneous TNFα level in vitro. Antibodies are detected at the admittance to the hospital by using HI assay, and the titers correspond to the inverse of the last dilution positive in inhibiting agglutination of goose red blood cells by dengue-2 antigen. Patients were designed as dengue primary-infected (black dots) or secondary-infected (white square) according to their HI titers in early and late sera (see text). Patients are segregated as high TNFα level (>142 pg/ml that is the mean + 2 SD of controls) or normal TNFα level in spontaneous heparinized whole-blood cultures compared with controls. The methods for heparinized whole blood cultures, TNFα measurements, virus isolation, RT-PCR, and HI assay are described in Patients, Materials, and Methods.

reaction occurs. Therefore, it can be produced spontaneously by cells in vitro but be undetectable in plasma, which can explain the results obtained in three patients in this study.

In contrast, heparinized whole blood from 13 patients secreted normal amounts of TNFα in vitro, whereas the levels of the molecule in the plasma of eight of the 13 were higher than those of controls. An increased level of TNFα produced in vitro may be masked by the presence of TNFα inhibitor. sTNFR is able to bind TNFα, but theoretically does not interfere in the immunologic assay used as stated above [Aukrust et al., 1994]. However, the possible interfering role of other unknown substances cannot be excluded. Indeed, recently Chang and Shaio (1994) reported the discovery of a new IL-1 inhibitor from the supernatant of dengue virus–infected monocytes. Moreover, the biomedical meaning of the measurements of cytokines produced spontaneously by whole-blood culture may be different and complementary to cytokine measurements in plasma.

The TNFα values in whole-blood cultures of patients stimulated with LPS and PHA were not higher than those of controls. It has been reported in septicemia and human immunodeficiency virus (HIV) infection that in vivo TNFα activation renders the cells less sus-

ceptible to further stimulation in vitro [Munoz et al., 1991; Steffen et al., 1993]. A decrease in stimulated TNFα release was not found in most patients with in vivo TNFα activation, i.e., high levels of TNFα in plasma and/or spontaneous in vitro production of TNFα. However, a low TNFα production was obtained in vitro in activated whole blood of three patients; in two of those patients, the spontaneous production of TNFα was normal, and virus genome was detected by RT-PCR in one of the two. Interestingly, Adler et al. [1996] demonstrated that a member of the Flaviviridae family downregulated the production of TNFα in macrophages in vitro. Due to the antiviral effects of TNFα and to the functions of TNFα in the activation of the immune response, a decrease in stimulated TNFα release in certain dengue-infected patients could facilitate the replication of the dengue virus and could contribute to the susceptibility of certain patients to various secondary infections.

Our results show that in most patients with detectable viremia, the level of TNFα was normal, whereas in patients without positive virus detection the levels of TNFα were high. The production of TNFα by human monocytes exposed to dengue 2 virus was recently reported [Chang and Shaio, 1994; Yang et al., 1995], but the mechanism of the raised levels of TNFα in dengue-infected patients has not been extensively studied. The data do not support the hypothesis of the role of circulating virus in the increased secretion of TNFα observed in patients. However, the role of a direct infection of monocytes or the role of soluble circulating dengue virus proteins in enhanced TNFα production cannot be excluded since exposure of monocytes/macrophages to dengue virus antigens induces TNFα production [Hober et al., 1996]. In addition, Kurane et al. [1991] detected high serum levels of IFNγ in dengue-infected patients, which could enhance the TNFα production by monocytes. Further investigation is required to define the role in vivo of infection of monocytes by dengue virus in the production of TNFα in patients.

The inverse correlation between TNFα production and viral replication in this study raises the question of the antiviral effect of TNFα in dengue infection. Indeed, TNFα is an antiviral molecule that may play a role in the attenuation of dengue virus infection [Mestan et al., 1986]; however, the rapid secretion of TNFα and other cytokines such as interleukin 1 (IL-1), IL-2, and non-cytokine chemical mediators such as platelet-activating factor (PAF), complement activation products C3a and C5a, and histamine can induce plasma leakage and shock which may be similar to those seen in patients with DHF [Kurane and Ennis, 1994]. The raised levels of immunoreactive TNFα in grade II and grade III dengue-infected patients in our study strongly indicate activation of the TNFα system during dengue infection. However, the mechanisms underlying the sensitization of a host to TNFα are not fully understood, and such influences together with virulence of dengue virus strain could explain the outcome of dengue infection in each patient [Pang, 1987; Wallach et al., 1990]. In order to elucidate whether the antiviral effect or pathophysiological role of TNFα predominates in dengue infection, studies of the variations of TNFα and virus isolation in sequential blood samples obtained consecutively for several days in dengue-infected patients should be performed.

A correlation was not found between pre-existing dengue virus antibodies and higher levels of TNFα in patients. Our results do not support the hypothesis of the role of antibody-dependent enhancement giving rise to increased viremic titers and production of TNFα [Anderson et al., 1997]. Kliks [1990] reported that virulent dengue 2 isolates associated with DHF/DSS and avirulent dengue 2 virus associated with dengue fever depend on enhancing antibodies to enter into monocytes, but monocyte infectivity may be modified further by factors related to the virus, such as viral uncoating. Together these results and our study do not invalidate the significance of antibody as a risk factor for severe clinical manifestations in dengue infection but suggest that viral virulence factors and host factors may in addition to antibodies be essential components for the pathogenesis of DHF/DSS.

Further investigations are needed to define the mechanisms of activation of TNFα and the role of this cytokine in dengue virus infection to improve the management of the disease.

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